Journal of Tissue Viability (2011) **20**, 20–29

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CLINICAL STUDY

The effects of soybean agglutinin binding on the corneal endothelium and the re-establishment of an intact monolayer following injury — A short review

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KEYWORDS
Corneal endothelium;
Wound repair;
Soybean agglutinin;
Migration;
Cell-contact;
Actin

Abstract This short review summarizes the localization and effects of the plant lectin soybean agglutinin (SBA) on the injured and non-injured organ-cultured rat corneal endothelium. Although the tissue exists as a non-cycling monolayer on the posterior corneal surface a circular freeze injury promotes wound repair as cells initiate DNA synthesis, mitosis and migration. As a result, by 24 h post-injury, endothelial cells express a surface protein that binds SBA in a diffuse punctate pattern, which by 48 h after injury, becomes confined to the cell periphery. As healing proceeds, SBA binding dramatically declines, such that, only scattered binding is observed by 72 h after wounding. In non-injured organ-cultured endothelia, weak SBA binding appears 24 h after explanation but becomes prominently detected around the cell periphery by 48 h. Incubating injured or non-injured endothelia in SBA leads to alterations in their cellular appearance due to the fact that lectin exposure results in the disruption of the actin cytoskeleton. Although this does not affect migration, treatment with either SBA or *N*-acetylgalactosamine (the SBA binding sugar) does interfere with the reestablishment of cell–cell contact. It is postulated that the surface protein that binds SBA is expressed during conditions that are stressful to the tissue. During organ-culture the protein's appearance suggests a cellular response to explantation in order to enhance or maintain monolayer integrity. In wound repair its appearance may serve to establish preliminary cell–cell contact during the restoration of the endothelial monolayer.

Introduction

The physiological function of any epithelium is dependent on the integrity of its cellular layer(s). When this situation is compromised, for example

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doi:10.1016/j.jtv.2009.11.008

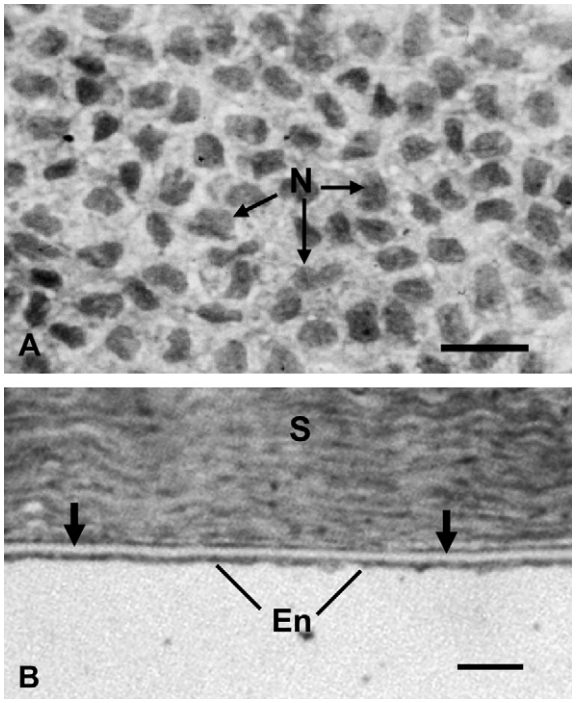

Figure 1 The rat corneal endothelium. Flat mount preparation (A), shows the normal corneal endothelium. Cells are arranged as a monolayer and display oval to kidney-shaped nuclei (N). In cross-section (B) the endothelium (En) adheres to Descemet's membrane (arrows) that attaches to the posterior surface of the corneal stroma (S).Bars = 50 μm (A) and 100 μm (B).

due to injury, the tissue loses the ability to carry out its normal functions which produce a disruption in homeostasis. In response to this type of situation, cells of the tissue initiate the process of wound repair that results in an orderly sequence of events such as DNA synthesis and mitosis leading to the healing of the damaged region and the restoration of normal tissue function.

In the vertebrate corneal endothelium the same scenario also exists. Cell damage to this tissue monolayer compromises its physiological function and results in decreased corneal transparency. In order to restore endothelial function, cells undergo repair processes such as mitosis and migration to re-establish both an intact monolayer as well as the normal physiological role of the tissue [1]. Interfering with wound repair *in vivo* using an inhibitor such as actinomycin D results in the inability of the endothelium to re-establish the monolayer and results in the persistence of increased corneal thickness, reflective of a compromise in endothelial function due to an incompletely restored monolayer [2]. In addition, inhibiting DNA synthesis with hydroxyurea still allows for cell movement into the wound but results in cell spreading as the major mechanism for restoring the monolayer. Upon removal of the drug, cells within the wound area reinitiate cell cycle traverse and complete mitosis to restore cell numbers to a level comparable to that seen in controls [2], indicating the importance of cell division to the endothelial repair process.

In this review the effects of the plant lectin soybean agglutinin (SBA) on rat corneal endothelial repair is discussed. This glycoprotein lectin has a molecular weight of approximately 120,000 D, an isoelectric point of close to 6.0 and consists of four subunits of nearly equal size with two binding sites for *N*-acetyl-D-galactosamine [3]. Although SBA binds preferentially to oligosaccarides having a terminal α- or β-linked acetylgalactosamine, it can also bind galactose residues to a smaller degree.

Work from our laboratory has indicated that rat corneal endothelial cells only bind SBA under conditions of stress, such as tissue explantation into organ culture or during wound repair. Cell surfaces of non-injured freshly isolated tissue fail to bind SBA [4], suggesting that the protein moiety responsible for this is absent from the cell surface under normal conditions. In support of this concept is the work of Panjwani and Baum [5] who did not observe SBA binding to isolated rabbit endothelial cell surface glycoproteins and Panjwani et al. [6] who could not detect SBA binding on histological sections using peroxidase histochemistry. Furthermore, work emanating from our laboratory has demonstrated that when SBA does bind to the surface of endothelial cells actin cytoskeletal organization, tissue morphology, and the re-establishment of endothelial monolayer integrity following wound repair are all affected.

The corneal endothelium

The endothelium of the vertebrate cornea (Fig. 1) resides on the tissue's posterior surface, resting along its basement membrane, Descemet's membrane, which it synthesizes [7]. Derived from neural crest cells [8,9], the tissue is a transport epithelium, involved in maintaining the hydration and thus transparency of the corneal stroma through a mechanism termed ''pump-leak'' [10]. This accounts for the fact that endothelial cells have incomplete tight junctions allowing fluid leakage of aqueous humor from the anterior chamber of the eye back into the stroma. The maintenance of stromal hydration is accomplished through the use of both Na^+/K^+ ATPases and bicarbonate pumps [11] located in the lateral cell membranes. Failure to maintain the intact endothelial cell layer compromises its pump function

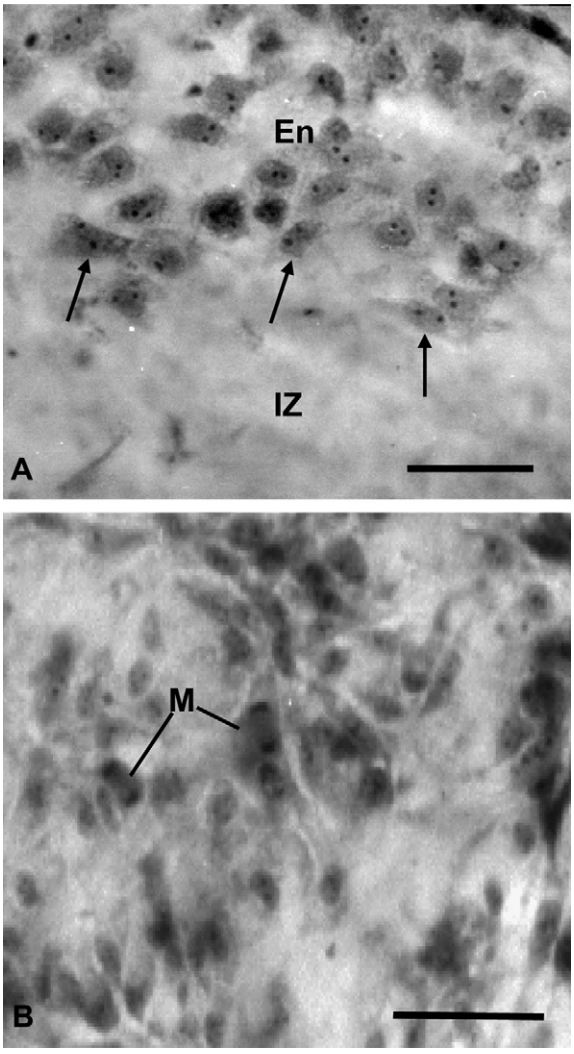

Figure 2 At 24 h post-injury (A), endothelial cells (En) around the injury zone (IZ) have lost cell—cell contacts with their neighbors but migration inward is minimal. At this time many cells immediately adjacent to the wound display elongated cellular process(arrows). By 48 h post-injury (B), the denuded area of the wound has filled in and mitotic cells are present (M) and the region exhibits disorganization. Bars = 50 μm.

and leads to a loss of corneal deturgescence and clarity.

During corneal development, endothelial cells actively undergo DNA synthesis [12—14] and cell proliferation [14,15] and then shortly after birth both processes abate, leaving the tissue comprised of cells that are in a state of proliferative quiescence [16,17], the so-called ''G_0'' phase of the cell cycle. As the tissue ages, some endothelial cells die, but instead of maintaining the cell population through mitosis, the surviving cells spread out and compensate for cell loss while allowing for the persistence of an intact monolayer in order to maintain stromal hydration. As a result, normal endothelial cell density decreases as a function of aging in vertebrates [18,19].

Endothelial wound repair

In contrast to the normal *in vivo* condition, injury to the corneal endothelium, such as what occurs following a circular freeze wound, causes increased transcriptional activity [20] and re-initiates cell cycle traverse and migration [16,17]. As such, cells surrounding the wound margin respond to the damage and restore the monolayer, whereas, cells on the tissue periphery do not partake in the repair process. In the rat, despite the fact that all endothelial cells are simultaneously in the G_0 phase of the cell cycle, wounding reveals that their response is not synchronous. Cells adjacent to the wound are stimulated to enter into the cell cycle first followed by those cells situated further away from the injury. As a result, DNA synthesis is observed in the tissue from 24 to 66 h post-injury and most mitoses occur between 42 and 66 h post-injury [17].

Besides a growth response, cells bordering the wound margin (Fig. 2A) also demonstrate a migratory response. As a result of a circular freeze injury, these cells find themselves adjacent to a region of denuded Descemet's membrane and soon begin to undergo morphological changes reminiscent of epithelial-mesenchymal transformation and begin to move into this area in order to re-establish a cell monolayer (Fig. 2B). Such movement occurs concurrently with the reorganization of the actin cytoskeleton. Initially actin is located around the cell periphery (Fig. 3A) as a circumferential microfilament band [2,14], but reorganizes as stress fibers in cells adjacent to the wound edge soon after injury [21] and subsequently are prominently observed in cells (Fig. 3B) that migrate into the damaged area [22—24]. As cells fill in the wound area, the region initially appears disorganized but eventually as repair processes (i.e., mitosis and migration) abate, the monolayer is restored although cells within the now repopulated area exhibit some degree of pleomorphism.

Interestingly, the migratory response to injury is also accompanied by the deposition of extracellular matrix protein despite the fact that endothelial cells are migrating across a natural extracellular matrix, Descemet's membrane. During this time, the cells display heightened levels and deposition of fibronectin and laminin [25,26] as well as thrombospondin [27], which is detected at the cell/matrix interface as distinct

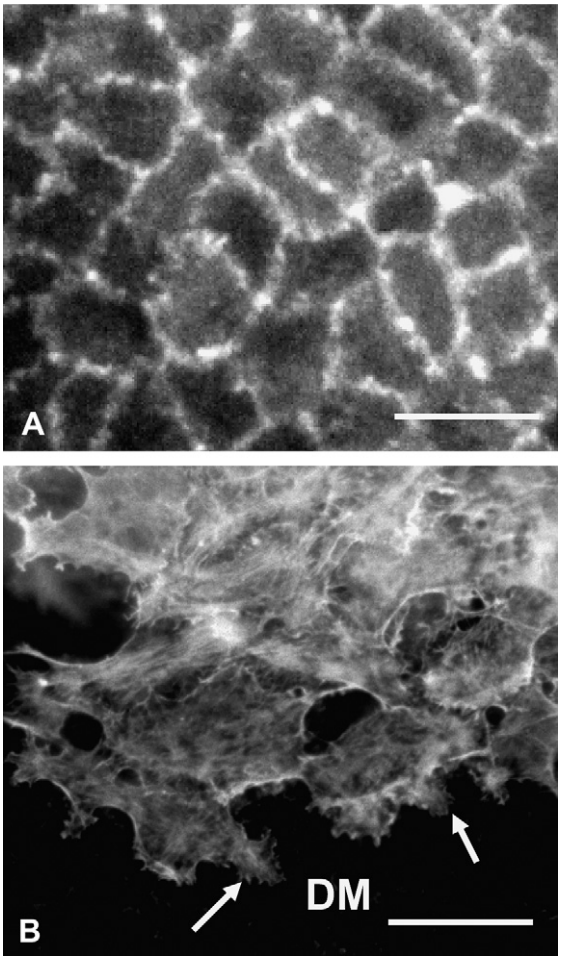

Figure 3 Fluorescence microscopy of actin patterns in the endothelium as detected by TRITC-phallodin staining. In (A), actin appears as a band around the periphery of each cell of the tissue. Twenty-four hours after a circular freeze wound (B), those cells adjacent to the injury have lost their pericellular actin pattern and now display stress fibers within their cytoplasm and processes (arrows). Bars = 40 μm (A) and 50 μm (B).

migration tracts, orientated in the direction of cell movement.

Soybean agglutinin binding to fetal endothelium

As previously mentioned, during gestation, endothelial cells are in a high state of growth, exhibiting both DNA synthesis and mitosis [12—15], a condition reminiscent of wound repair in the adult. As gestation proceeds, both processes slowly decline so that shortly after birth, both abate in the endothelium [14], thus establishing the growth limitations for the adult tissue. Concurrent with this cessation of growth is the establishment of the pericellular circumferential microfilament bundle [14] that imparts an actin honeycomb appearance that is characteristic of this cell layer [24]. In all probability, as is the case for other epithelial type tissues, the establishment of this microfilament band is crucial to the formation of the intact tissue monolayer, via the formation of the zonula adherens junctions [28—30]. During the latter part of development, fetal endothelial cells exhibit surface binding of the SBA lectin. When examined with fluorescence microscopy, rat endothelial cells at 16 days of gestation bind SBA. At this point in their development, the tissue is mitotically active but has yet to exhibit a well-defined monolayer. However, by the 19th day of gestation, the monolayer is much better defined, mitosis has greatly diminished [14] and SBA binding can no longer be detected on the cell surfaces of these late stage embryos [4]. Thus, at a time when endothelial cells display vigorous cell growth but do not demonstrate a well-defined monolayer, SBA binding is readily observed. On the other hand, as the monolayer formation becomes more established, lectin binding disappears from the cell surface. In this regard, SBA binding resembles the situation observed during adult wound repair, whereas the lack of SBA binding in the later organized fetal endothelium mimics the scenario seen in the non-injured adult tissue.

Soybean agglutinin binding during endothelial wound repair

When rat corneal endothelia are given circular freeze injuries, cells around the wound area respond by initiating cell cycle traverse and begin to migrate into the wound region in order to repopulate this zone and restore an intact and functional monolayer. Intact endothelia of non-injured tissues fail to bind SBA as determined by fluorescence microscopy, but show SBA binding in response to injury. The fact that the binding occurs due to the synthesis of a cell surface SBA-binding protein was shown in experiments involving puromycin, which resulted in the absence of SBA binding to endothelial cell surfaces [31]. By 24 h post-injury (Fig. 4A) the binding appears light and as a punctate speckling over the entire cell surface, but over the next 24 h, as repair progresses, the binding intensifies and now becomes more localized to the cell periphery [4,31], so that by fluorescence microscopy it appears to outline cells within the injury zone (Fig. 4B). At this time (48 h post-wounding), the damaged area has been repopulated with cells and the monolayer is starting to become re-established. As the reorganized monolayer becomes

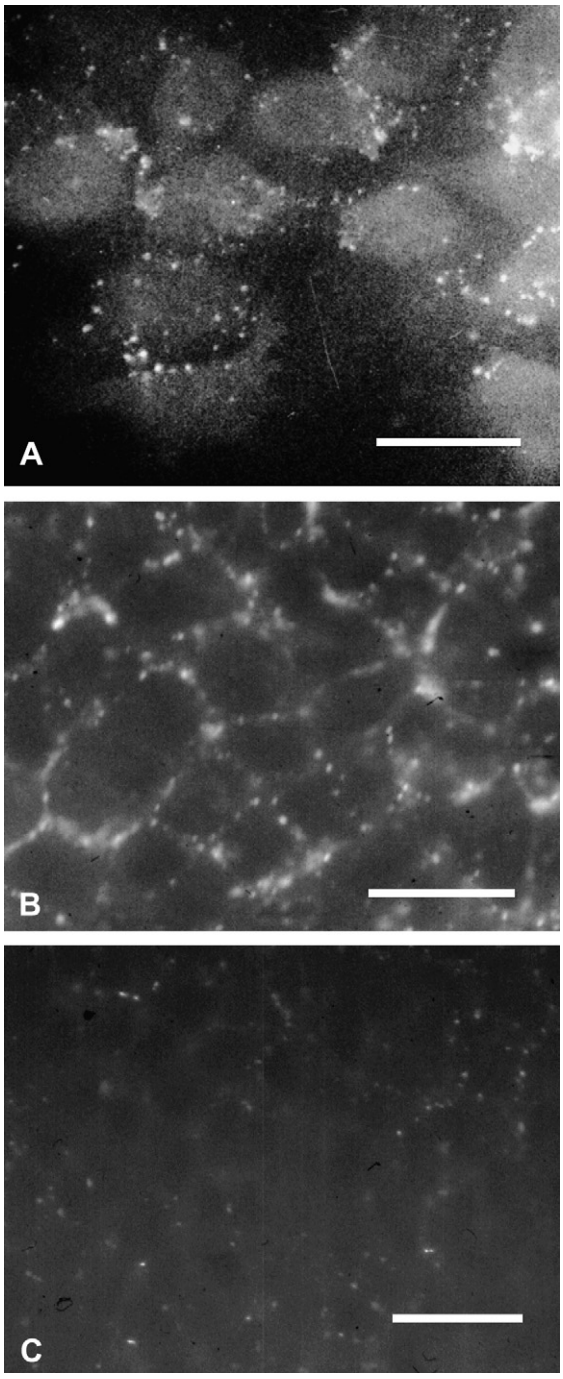

Figure 4 Fluorescence microscopy of soybean agglutinin (SBA)-FITC binding in the injured endothelium. Cells surrounding the wound 24 h after injury (A), display a punctate binding pattern over their surfaces. By 48 h post-injury (B), cells that have repopulated the injury zone now display SBA binding in a region confined to their periphery, so that the pattern outlines the cells. By 72 h post-wounding (C), cells within the wound area demonstrate a precipitous drop in SBA binding to their surfaces. Bars = 40 μm (A, B) and 50 μm (C).

restored, the levels of SBA that bind to the tissue decrease precipitously by 72 h post-injury (Fig. 4C), such that after one week post- injury only very sparse and scattered binding can be detected [4]. This result is interpreted to indicate that the appearance of the SBA binding protein on the endothelial cell surface is a transient event and that once the restoration of the monolayer is accomplished, this surface protein and with it, SBA binding, essentially disappears. A similar finding was also observed in studies on embryonic Xenopus muscle cell adhesion *in vitro* where SBA localization was shown to be initially accumulated at sites of contact [33], and then progressively decreased over a 24 h period.

Actin disruption following soybean agglutinin binding

When responding to a circular freeze injury, endothelial cells lose their cell—cell connections with their adjacent neighbors. Early indications of this change are observed by 2 h post-wounding as cells around the injury demonstrate a loss of CMBs but begin to display extensions of cell processes into the region of the now denuded Descemet's membrane [21]. As a requirement of migration, these cells, as mentioned before, also undergo a morphological transformation usually referred to as an epithelial/mesenchyme transition [34] and take on a fibroblastic-like appearance as they move into the wound area. Unlike other epithelial cell types that characteristically migrate as a sheet [35—38], endothelial cells have a propensity to migrate as individual cells that is no doubt derived from their neural crest cell origins. As already stated above, reorganization of the actin cytoskeleton accompanies their migratory response as the cell's circumferential microfilament band disappears and the cytoplasm becomes filled with actin containing stress fibers [21,23,39]. This actin pattern change occurs due to a reorganization of the pre-existing actin within the band and occurs in the presence of actinomycin D, a DNA-directed RNA synthesis inhibitor, and without benefit of any actin synthesis, as measured by a lack of methionine-^{35}S incorporation into immunoprecipitated actin [22].

When injured endothelia are placed into organ culture and exposed to100—200 μg/ml SBA there is no affect on either mitosis or migration [32] and wound repair occurs at the same rate as in control preparations. However, migrating cells no longer display the broad extended cell processes observed in their control counterparts. Many of the cells have slender processes extending from either end of their

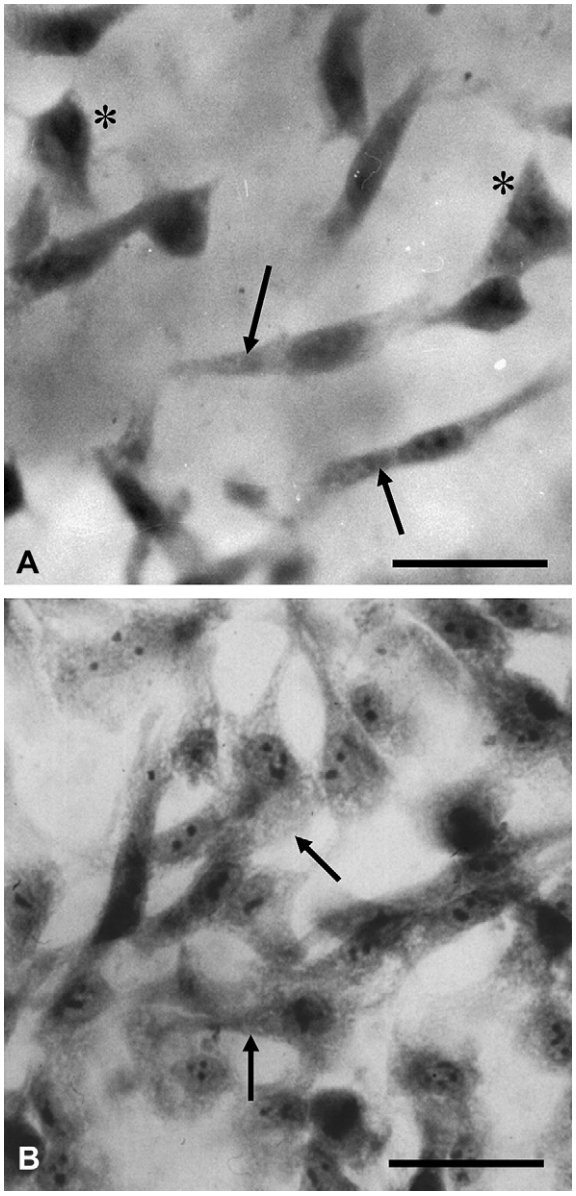

Figure 5 When injured endothelia are placed into organ-culture in the presence of 200 μg/ml SBA their morphology is altered. Many of the cells (A) now display slender process (arrows) that extend outward from either side of the cell body, while others (asterisk)appear pyramidal in their overall shape. Cells in control preparations (B) demonstrate broad processes (arrows) extending from their cell bodies. Bars = 40 μm.

cell bodies (Fig. 5). Still, other cells appear pyramidal and their processes have been shown to end rather bluntly [31]. Examination using fluorochrome-conjugated phallotoxins revealed that SBA treatment resulted in a disruption of actin organization (Fig. 6 A), so that migrating cells no longer displayed their usual (Fig. 6B) stress fiber patterns [32]. In addition, in the presence of SBA, the circumferential microfilament band in cells of non-injured tissue also became disrupted (Fig. 6C), and this resulted in the loss of endothelial monolayer integrity due to the disruption of cell—cell and cell/matrix adhesion [31]. Thus, SBA binding to the endothelium results in a total disorganization of the actin cytoskeleton in both the injured and non-injured tissue. This effect of SBA on actin cytoskeletal organization has also been noted in other systems [40,41], but whether this involves an interaction with the same cell surface protein as in the endothelium is as of yet unknown.

In contrast to the above results, when tissues were pre-incubated in the presence of 1—2 μM of phallacidin overnight and then exposed to the lectin, the effects of SBA on actin disorganization was limited. Under this condition, where phallacidin served to stabilize actin filaments, cells displayed some retention of their circumferential microfilament band even after 48 h in SBA containing medium [31]. Conversely, in control samples exposed to SBA, not only were the circumferential microfilament bands completely disrupted, the effect was not readily reversible even after 48 h in SBA-free medium, suggesting that the lectin effect on the bands involved more than a simple depolymerization of their microfilaments [31]. Interestingly, unpublished observations from our laboratory indicate that although SBA can disrupt the organization of the actin cytoskeleton, depolymerizing the microfilaments with cytochalasin B treatment prior to lectin incubation resulted in no apparent change in the localization of SBA binding, suggesting that any link existing between the SBA-binding protein and the actin cytoskeleton must be one of an indirect nature.

Wound repair in the presence of soybean agglutinin

As previously stated, the presence of SBA does not interfere with endothelial wound repair. Cells undergo their mitotic and migratory responses and the tissue monolayer becomes re-established at the same rate as does control tissues [32]. In studies on chick cardiac mesenchyme cells, exposure to 100 μg/ml SBA likewise had no effect on *in vitro* migration [42] and results reported by Gipson and Anderson [43] also indicated that SBA treatment failed to decrease the migratory response of corneal epithelial cells following a central abrasion wound to the tissue. Thus, SBA in and by itself does not appear to have any effect on, nor interfere with, growth and migratory responses.

However, results from our laboratory indicate that SBA exposure does disrupt cell—cell adhesion

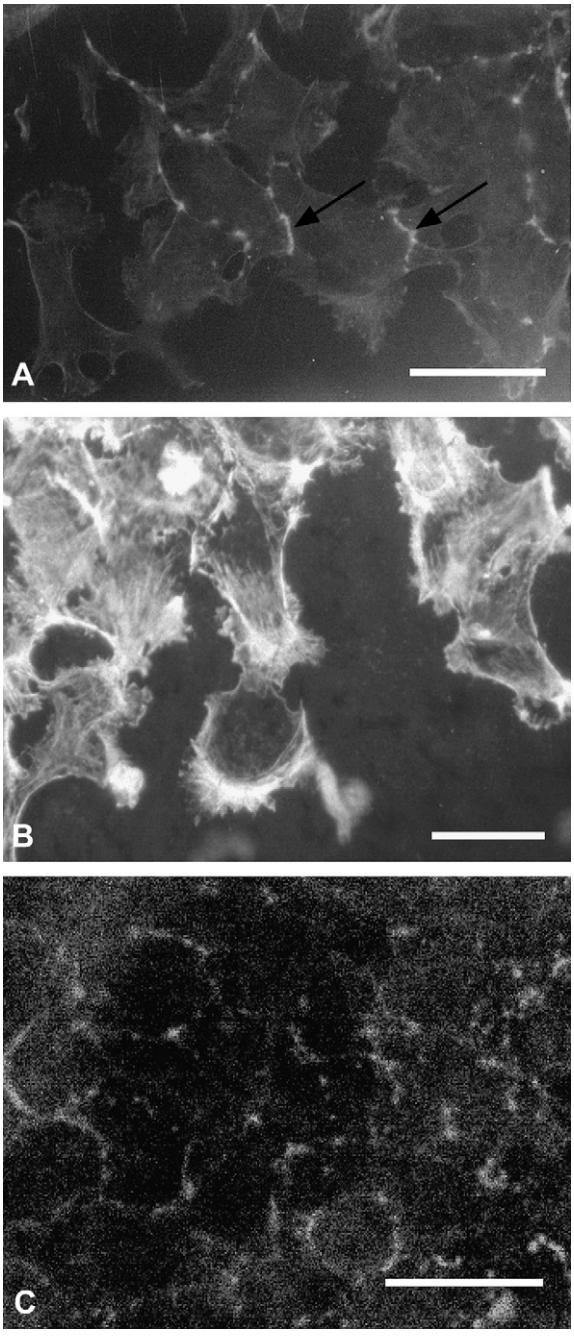

Figure 6 Incubation of endothelial tissue in soybean agglutinin (SBA) at a concentration of 100 μg/ml promotes disorganization in the actin cytoskeleton as detected by phalloidin-FITC staining. In (A), cells surrounding the wound area extend processes into the wound but do not display any organized actin pattern as stress fibers or microfilaments. In some instances, actin remnants appear as clumps along areas of cell contact (arrows). In control preparations (B), microfilaments are readily observed. When non-injured endothelia are placed into organ-culture in the presence of 100 μg/ml SBA (C), their circumferential microfilament band becomes severely disrupted after 48 h. Bars = 40 μm.

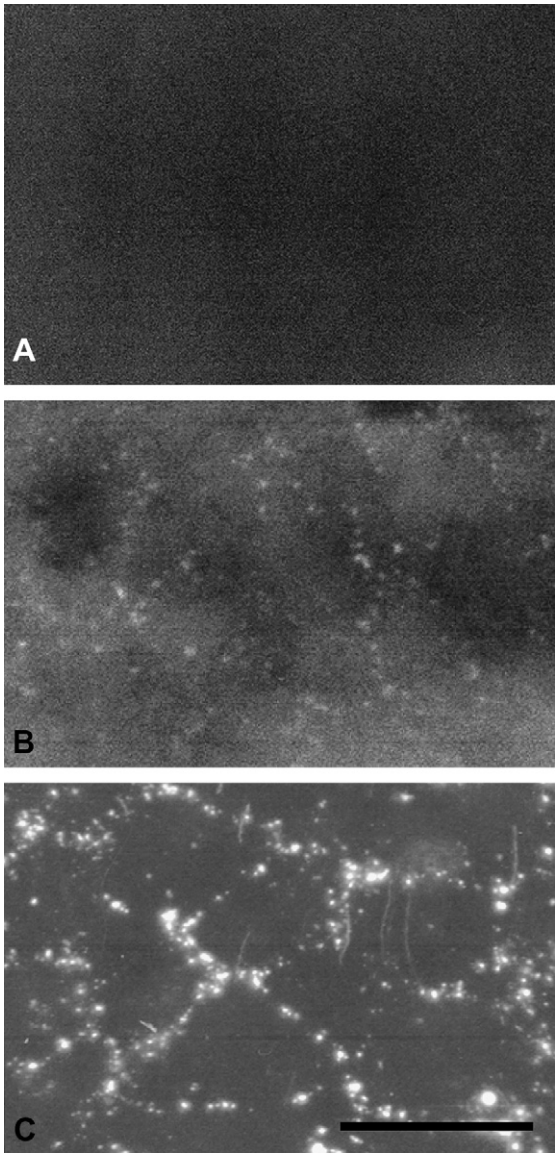

Figure 7 Explantation of non-injured corneal endothelium into organ-culture promotes the expression of soybean agglutinin (SBA) binding. After a 6 h incubation (A) endothelia fail to demonstrate any SBA binding on their cell surfaces. By 24 h (B) a small amount of SBA binding is now observed along regions of cell—cell interfaces. When tissues are examined after 48 h in culture (C), increased levels of SBA binding are now easily detected around the cell periphery, along areas of cell—cell contact. Bar = 40 μm (A, B and C).

in the non-injured endothelium and prevents the re-establishment of cell—cell contacts during wound healing [31]. SBA does not bind to freshly isolated and fixed non-injured tissue, indicating that the major SBA binding sugar, *N*-acetylgalactosamine is absent on the surfaces of endothelial cells *in vivo*. Nonetheless, when non-injured endothelia are explanted into organ culture, (a

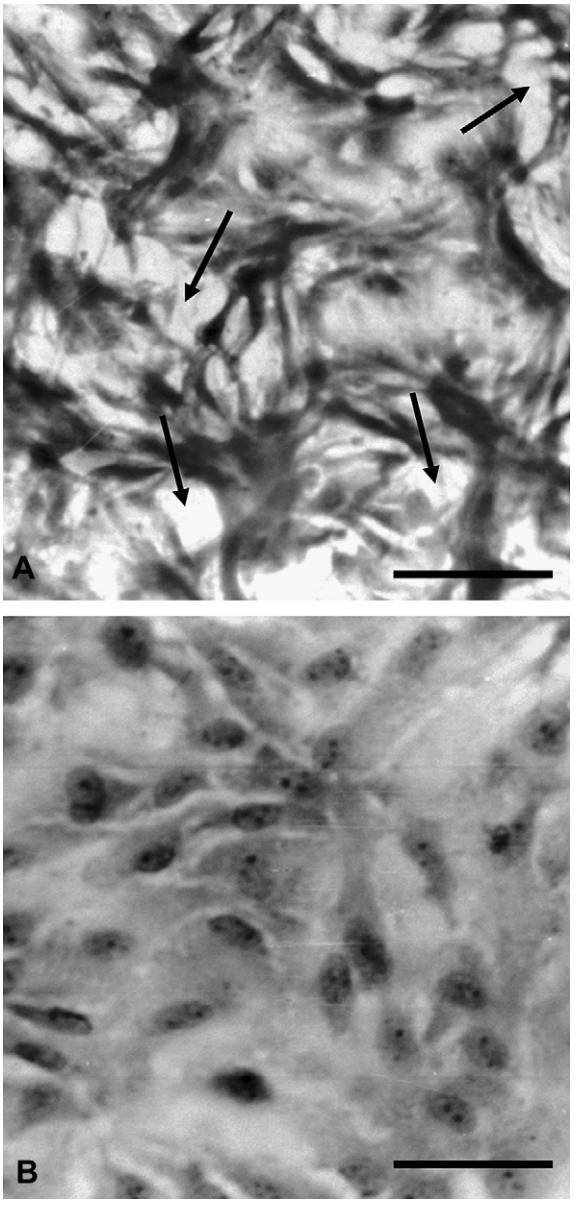

Figure 8 The effects of *N*-acetylgalactosamine on endothelial wound repair. When injured endothelia are culture in the presence of the sugar, the wound area (A) remains disorganized 72 h post-injury. Although cells migrated into the region, they have not established the cell—cell contacts nor begun to restore an intact monolayer as do control tissues (B). Bars = 40 μm.

form of stress for the tissue) the cells will subsequently exhibit the ability to bind SBA. After an incubation period of 6 h, no SBA binding can be detected by fluorescence microscopy (Fig. 7A). However, by 24 h after explantation the non-injured tissue exhibits light SBA binding along it's cell borders (Fig. 7B), and by 48 h post-explantation, the binding has become intense and obviously restricted to a region around the cell periphery (Fig. 7C), so that it essentially outlines the cell boundary. As already noted, in wounded endothelia, by 24 h post-injury, cells around the injury zone display a scattered punctate pattern of SBA binding on their cell surface. As the monolayer repairs itself and cells undergo interactions in order to re-establish the monolayer the lectin pattern also becomes confined to the cell periphery [32]. This SBA binding pattern co-localizes with the junctional proteins ZO-1 and *N*-cadherin [31], and suggests that this peripheral localization may well reflect that the protein binding the lectin plays a role in cell—cell adhesion. Strengthening this concept are results that demonstrate the presence of SBA during wound repair impairs the re-establishment of close cell—cell contact as the wound area is repopulated [31]. Interestingly, studies on *Xenopus* myotomal muscle cells *in vitro* has shown that when they formed contacts with one another SBA receptors became highly concentrated in the regions of adhesion [33]. Whether or not the SBA receptor protein in these cells is similar to that in the endothelium remains to be elucidated by further studies.

In addition to using SBA to interfere with cell—cell adhesion, incubation of the tissue in *N*-acetylgalactosamine [44] also proved capable of inhibiting cell contact. Although the cells migrated into and filled the wound area, the presence of this competing sugar resulted in the appearance of gaps around the cells and caused a retardation in reestablishing an intact monolayer (Fig. 8). Furthermore, incubation in *N*-acetylgalactosamine also resulted in a loss of ZO-1 immunostaining in the cells migrating in response to the injury [31]. Similarly, Chow and Poo [33] also demonstrated in Xenopus embryonic myotomes that cell contact in culture was decreased in the presence of *N*-acetylgalactosamine.

The prevention of cell—cell contact in the presence of *N*-acetylgalactosamine suggests that the SBA binding protein may function via homophilic interactions with other endothelial cells. During development, homotypic cell types use selective intercellular adhesion for the morphogenesis of embryonic tissue. In the corneal endothelium, the cellular events associated with wound repair mimic those of the developing tissue [4], suggesting that when tissues undergo wound repair they may, to some degree, revert back to an embryonic state in which many of the cellular processes used in healing essentially mimic those observed during development. In support of this are studies that demonstrate fibronectin splicing during dermal wound repair in the rat resembles

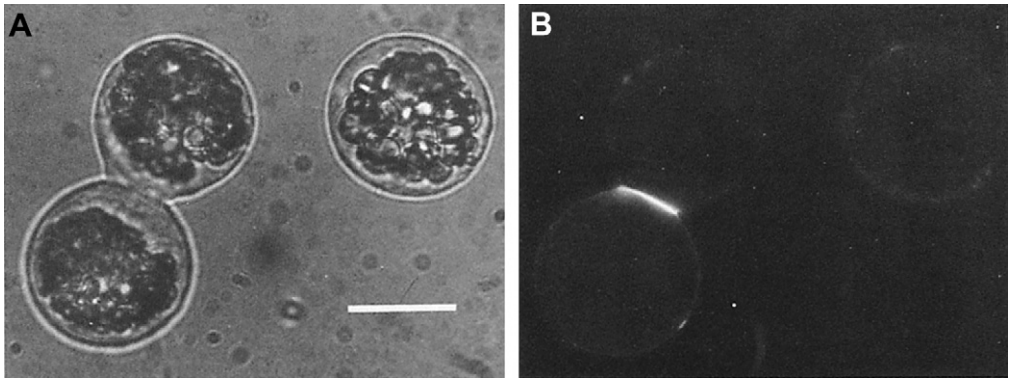

Figure 9 Soybean agglutinin (SBA) binding to cultured *Xenopus* embryonic heart muscle cells following contact with each other. Phase-contrast microscopy (A) of two cells in contact with one another and the corresponding fluorescence micrograph (B) showing SBA localization at the interface between the two cells. Bar = 20 μm. Reprinted from Chow and Poo [33]. © Chow I. and Poo M.-M., 1982. Originally published in *Journal of Cell Biology*. 95:510—518.

that observed in the embryonic state [45] and that at the cell level many of the mechanisms used for wound repair appear to be conserved [46,47]. Thus, it is possible that in the corneal endothelium, those cells that partake in healing, revert back to an embryonic-like condition and proceed to carry out wound repair using mechanisms similar to that of embryonic systems in order to re-establish the intact tissue monolayer.

The final stage in epithelial wound healing must ultimately be the re-adhesion of adjacent neighboring cells to restore an intact cell layer(s). Because endothelial cells lose contacts with their neighbors in order to migrate as individual cells into the wound, the subsequent restoration of these contacts is critical for both the reformation of the monolayer and the eventual return of the tissue's physiological function. The role of an SBA binding protein may be inferred by its transient appearance during wound repair. It can be hypothesized that the presence of this protein is responsible for the initial stages of cell—cell recognition and/or adhesion and that it serves to form a temporary linkage between endothelial cells until they can re-establish their junctional complexes. In support of this concept, studies by Chow and Poo [33] demonstrated not only a clustering of SBA receptors along areas of contact (Fig. 9), but a progressive decrease in their presence over a 24 h time period. This interaction resulted in the cells acquiring a greater adhesion toward one another as determined by separation using micromanipulation techniques. Thus, further studies on this SBA-binding protein are required that will allow for an elucidation of its role in re-establishing cell—cell contact during corneal endothelial wound repair, and perhaps in other systems as well.

Conflict of interest statement

None

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